Prion 3:4, 236-239; October/November/December 2009; © 2009 Landes Bioscience

In vitro amplification of prions from milk in the detection of subclinical infections

Kevin C. Gough,[1,*] Claire A. Baker,[2] Maged Taema[1] and Ben C. Maddison[2]

[1]School of Veterinary Medicine and Science; The University of Nottingham; Sutton Bonington, Leicestershire UK; [2]ADAS UK; Department of Biology; University of Leicester; Leicester, UK

Prions can be amplified by serial protein misfolding cyclic amplification (sPMCA) from the milk of a high proportion of apparently healthy, scrapie exposed sheep with *PRNP* genotypes not previously associated with high disease penetrance.[1] These data strongly suggest the widespread presence of subclinical scrapie infections within scrapie-exposed flocks containing sheep with a range of susceptible *PRNP* genotypes. These data also lead to the hypothesis that similar subclinical disease states may be common for other animal and human prion diseases. Furthermore, the application of sPMCA to milk provides a method to detect such subclinical disease. Here, we describe the high level amplification of bovine spongiform encephalopathy (BSE) prions from both ovine and bovine origin, a methodology that will facilitate the detection of any prions secreted within bovine and ovine milk during subclinical and clinical BSE disease.

Key words: scrapie, prion, PrP, BSE, milk

Submitted: 06/30/09

Accepted: 10/23/09

Previously published online: www.landesbioscience.com/journals/prion/article/10425

*Correspondence to: Kevin C. Gough; Email: kevin.gough@nottingham.ac.uk

Serial protein misfolding cyclic amplification (sPMCA) involves the amplification of minute quantities of PrP^{Sc} seeded into PrP^{C} substrate using iterative rounds of sonication and incubation at 37°C, and offers exquisite sensitivity for the detection of prions. This technique was pioneered by Soto and colleagues[2,3] and has been used to detect prions in both blood[2] and brain material[3] in the preclinical stages of rodent-scrapie. Importantly, this methodology has been applied to the high level amplification of PrP^{Sc} from natural hosts of prion diseases including human variant-Creutzfeldt-Jakob disease (vCJD),[4] cervine chronic wasting disease (CWD)[5] and ovine scrapie.[6] Together, data suggests that sPMCA has obvious application to the monitoring of prions within subclinical disease. Recently, sPMCA was applied within our laboratory to detect PrP^{Sc} within ovine milk secreted from sheep incubating natural scrapie infections.[1] We reported the presence of prions within milk of clinically normal sheep with a range of *PRNP* genotypes, AHQ/VRQ ($AV_{136}HR_{154}QQ_{171}$), ARQ/VRQ and ARR/VRQ. The AHQ/VRQ and ARR/VRQ *PRNP* genotypes are typically associated with relatively limited disease penetrance.[7] This data strongly suggests the detection of subclinical disease in some of these animals and highlights the analysis of milk as a suitable matrix to measure subclinical prion diseases. Given that cervid CWD, human vCJD, and experimental ovine BSE display similar tissue tropism of PrP^{Sc} compared to ovine scrapie, it seems possible that an analogous secretion mechanism occurs for these other prion diseases and that the sPMCA analysis of milk could facilitate the detection of subclinical disease states.

A subclinical state for prion diseases has been defined as the replication of prions that does not lead to clinical disease within the normal life span of the host.[8] Where such a definition is applied to food chain animals such as cattle and sheep, the normal life span could be defined as the age at slaughter. The occurrence of subclinical scrapie in sheep has been described several times in the literature[9-11] where the presence of subclinical infections within a flock with a high

proportion of scrapie-susceptible *PRNP* genotypes far outnumbered those animals with clinical disease. Similarly, subclinical BSE infections have been reported in cattle[12,13] where infectivity is detected within Peyer's patches and tonsils and then later within the sciatic nerve and brainstem of apparently healthy cows.[13] With respect to experimental models of prion diseases, several reports describe the presence of subclinical disease as a result of both between- and within-species transmissions. The inoculation of mice with high doses of hamster scrapie prions has been shown to produce demonstrable replication of prions without the advent of clinical disease.[8,14] In addition, inoculation of rodents with low doses of rodent-adapted prion strains originating from ovine and human sources has been demonstrated to produce subclinical infections.[15-18] Such studies have demonstrated the production of subclinical infection following oral challenge and that the presentation of subclinical, as opposed to clinical disease, is dictated by the levels of PrP within the host animal as well as the infectious dose.[16] The advent of mice transgenic for the *PRNP* gene has yielded in vivo models to investigate the ability of different PrP proteins to sustain the replication of various prion strains. The use of mice transgenic for human *PRNP* sequences coding for either MM or MV at codon 129 demonstrated the common occurrence of subclinical infections as a result of inoculation with human vCJD or bovine BSE prions.[19,20] Overall, accumulating evidence indicates that subclinical prion infections may well be a common outcome after exposure to the prion agent.

In all instances, the ability to detect a subclinical prion disease will be dependent upon the sensitivity of the method employed to detect either PrP^{Sc} or prion infectivity. Studies have compared the relative sensitivities of methodologies in this regard and have concluded that immunohistochemistry and histopathological examination of the medulla oblongata as well as western blotting of cerebellar tissue provided similar sensitivities.[9] Compared to these conventional western blotting and histopathological methods, the inclusion of sodium phosphotungstic acid precipitation of PrP^{Sc} within the western blot protocol was shown to improve assay sensitivity, and bioassay using mice overexpressing murine PrP was 10–100-fold more sensitive still.[17] Within a rodent model system, the latter was able to routinely demonstrate subclinical infections in mice challenged with low doses of inoculum, which were clinically normal and did not contain detectable PrP^{Sc} using the above immunological and histopathological techniques.[17] Whilst highly sensitive and specific, the routine application of mouse bioassay to monitor for subclinical infections within ruminants would be impractical due to time and ethical considerations. An obvious, alternative methodology for the detection of subclinical prion infections in ruminants is provided by sPMCA which is at least as sensitive as mouse bioassay.[21]

Our previous study applying sPMCA to ovine milk demonstrated the potential of this methodology to screen for subclinical scrapie infections. This study also demonstrated the secretion of prion in ovine milk irrespective of the immunological detection of PrP^{Sc} within the animal's LRS. Furthermore, prion was also detected in milk from animals with ovine *PRNP* genotypes not typically associated with LRS involvement in PrP^{Sc} replication, namely AHQ/VRQ and ARR/VRQ.[22] Such limited LRS involvement in prion propagation is similar to that reported in bovine BSE; this data therefore indicates that an analogous secretion of low levels of prion within milk may occur with subclinical and clinical bovine BSE infections. To date, the PMCA methodology has allowed only a relatively modest 32-fold amplification of bovine BSE.[23] Here, we demonstrate that both bovine and ovine BSE prions are readily amplified to high levels by sPMCA. This was achieved within both bovine brain homogenate and when using ovine PrP^{C} from a sheep with an ARQ/ARQ *PRNP* genotype (**Fig. 1A**); an ovine genotype known to be favoured for BSE replication in vivo.[24,25] Bovine BSE prions were routinely amplified 1,000-fold in ovine brain homogenate substrate after four rounds of sPMCA (**Fig. 1A**). Amplification of ovine BSE prions within the same ovine substrate was achieved to 300,000-fold after four rounds of sPMCA (**Fig. 1A**). Additionally, both ovine BSE and bovine BSE could be amplified with similar efficiencies when spiked into unpasteurised whole milk and using five rounds of amplification (**Fig. 1B**). These amplifications were comparable with those for classical scrapie prions using an ovine PrP^{C} substrate with a VRQ/VRQ *PRNP* genotype (**Fig. 1**). It should also be noted that the in vitro replication of BSE prions maintains the characteristic molecular phenotype of this prion strain (**Fig. 1C**). These data clearly demonstrate that sPMCA facilitates the high sensitivity detection of ovine BSE and bovine BSE prions and the method is applicable to the analysis of ruminant milk. This method could be used to determine whether BSE prions are secreted in ruminant milk and to monitor for subclinical BSE disease states within ruminants.

Bovine milk is an important component of the human diet as well as animal feeds. Given the BSE epidemic in the UK and the continuing occurrence of low numbers of BSE cases, if prions are secreted within bovine milk in a mechanism analogous to that found in subclinical and clinical scrapie it seems most probable that considerable numbers of people and ruminants have been exposed to the BSE agent through milk ingestion. Individuals would likely to be exposed to very low levels of prions but on numerous occasions. Descriptions of the effects of repeated challenge with a prion agent are very limited and provide contrasting results. Diringer and co-workers used a hamster scrapie model and demonstrated that whilst the risk of disease was greater upon repeat oral exposure to low doses of prions, this was to a lesser extent than if the total cumulative dose was used in a single oral challenge.[26,27] In contrast, Jacuemot and co-workers used a mouse scrapie model and an intraperitoneal route of inoculation and found that mice receiving repeated doses of low infectivity had much higher incidences of disease than mice injected once with the same cumulative dose.[28] The reasons for these contrasting results is unclear but may be contributed to by differences in the prion strain, host factors, inoculum preparation, challenge schedule and the route of inoculation. What is certain is that the pathogenesis of prion diseases resulting from repeated low dose exposure is poorly understood.

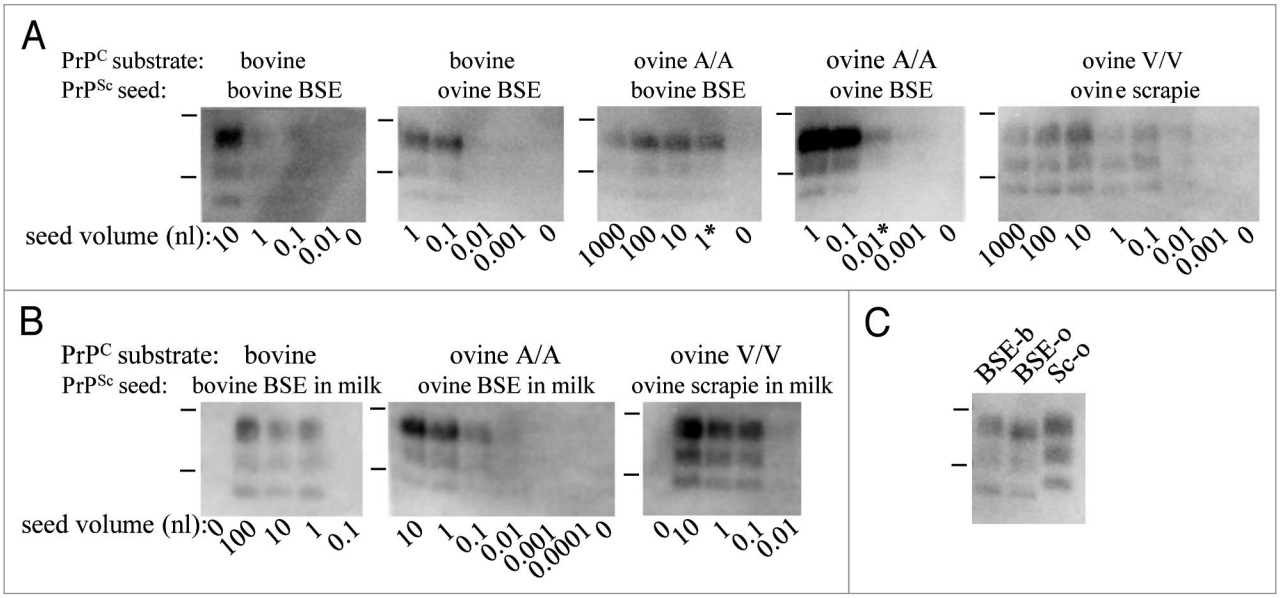

Figure 1. sPMCA analysis of ruminant prions. Serial 10-fold dilutions of 10% (w/v) brain homogenate from clinically affected sheep with either scrapie or BSE (A and B as indicated), or cattle with clinical BSE (A and B as indicated) were seeded into sPMCA reactions (seeds contained 1,000 to 0.0001 nl of 10% brain homogenate per gel lane as indicated; reactions containing no PrP^{Sc} seed are also shown). Serial PMCA substrates were 10% (w/v) brain homogenates from TSE-free bovine or ovine sources. The latter were animals with either VRQ/VRQ (V/V) or ARQ/ARQ (A/A) *PRNP* genotypes (as indicated). Each sample underwent either four (A) or five (B) rounds of sPMCA and products were digested with proteinase K before analysis of 10 μl of each sample on western blots. PrP was detected with monoclonal antibodies SHA31 and P4 and molecular weight markers of 30 and 20 kDa are indicated. Brain homogenate seed were added directly into sPMCA reactions (A) or were first spiked 1 in 10 into unpasteurised whole milk (B). Milk has obtained from TSE-free animals and processed for sPMCA as described by Maddison and co-workers;[1] all sPMCA reactions were also carried out as described previously.[1] Bovine BSE was amplified and detected from 1 nl of brain homogenate seed and ovine BSE from 0.01 nl (lanes marked with an asterisk, A). The direct western blot analysis of 1 μl and 3 μl of the bovine BSE and ovine BSE 10% brain homogenates respectively produced detectable levels of PrP^{Sc} (data not shown) indicating amplifications of 1,000-fold and 300,000-fold for bovine BSE and ovine BSE respectively by sPMCA. Bovine BSE amplified by sPMCA in bovine brain homogenate substrate (C, BSE-b) and ovine BSE amplified by sPMCA in ovine brain homogenate substrate (C, BSE-o) both produced PK-resistant PrP^{Sc} fragments that displayed lower molecular weights than analogous fragments produced with ovine scrapie amplified by sPMCA in ovine brain homogenate substrate (C, Sc-o).

Evidence from previous epidemiological and bioassay studies would suggest that any presence of BSE prions within bovine milk does not lead to efficient transmission of clinical disease.[29,30] However, it cannot be discounted that prions are present within bovine milk at levels too low to cause clinical disease. Also, as mentioned above, the exposure of animals to low levels of prions is one factor that can lead to subclinical disease states and furthermore, it is unclear what effects repeat exposure with low dose inoculum may have on prion propagation and pathology. Given the importance of milk to animal and human food chains, it seems of high importance to apply up to date high sensitivity methods such as sPMCA to determine whether prions are secreted within bovine milk and take advantage of this readily available biological matrix to study subclinical disease states for ruminant prion diseases.

References

1. Maddison BC, Baker C, Rees HC, Terry LA, Thorne L, Bellworthy SJ, et al. Scrapie prions are secreted in ovine milk from clinically normal scrapie-exposed animals. J Virol 2009; 83:15-8.
2. Saá P, Castilla J, Soto C. Presymptomatic detection of prions in blood. Science 2006; 313:92-4.
3. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 2001; 411:810-3.
4. Jones M, Peden AH, Prowse CV, Gröner A, Manson JC, Turner ML, et al. In vitro amplification and detection of variant Creutzfeldt-Jakob disease PrP^{Sc}. J Pathol 2007; 213:21-6.
5. Kurt TD, Perrott MR, Wilusz CJ, Wilusz J, Supattapone S, Telling GC, et al. Efficient amplification of chronic wasting disease PrP^{RES}. J Virol 2007; 81:9605-8.
6. Thorne L, Terry LA. In vitro amplification of PrP^{Sc} derived from the brain and blood of sheep infected with scrapie. J Gen Virol 2008; 89:3177-84.
7. Tongue SC, Pfeiffer DU, Warner R, Elliot H, Del Rio Vilas V. Estimation of the relative risk of developing clinical scrapie: the role of prion protein (PrP) genotype and selection bias. Vet Rec 2006; 158:43-50.
8. Hill AF, Joiner S, Linehan J, Debruslais M, Lantos PL, Collinge J. Species barrier independent prion replication in apparently resistant species. Proc Natl Acad Sci USA 2000; 97:10248-53.
9. Thorgeirsdottir S, Georegsson G, Reynisson E, Sigurdarson S, Palsdottir E. Search for healthy carriers of scrapie: an assessment of subclinical infection of sheep in an Icelandic scrapie flock by three diagnostic methods and correlation with PrP genotypes. Arch Virol 2002; 147:709-22.
10. Georgsson G, Adolfsdottir JA, Palsdottir A, Jorundsson E, Sigurdarson S, Thorgeirsdotir S. High incidence of subclinical infection of lymphoid tissues in scrapie-affected sheep flocks. Arch Virol 2008; 153:637-44.
11. Ersdal C, Ulvund MJ, Benestad SL, Tranulis MA. Accumulation of pathogenic prion protein (PrP^{Sc}) in nervous and lymphoid tissues of sheep with subclinical scrapie. Vet Pathol 2003; 40:164-74.
12. Schaller O, Fatzer R, Stack M, Clark J, Cooley W, Biffiger K, et al. Validation of a western immunoblotting procedure for bovine PrP^{Sc} detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). Acta Neuropathol 1999; 98:437-43.
13. Espinosa JC, Morales M, Castilla J, Rogers M, Torres JM. Progression of prion infectivity in asymptomatic cattle after oral bovine spongiform encephalopathy challenge. J Gen Virol 2007; 88:1379-83.
14. Race R, Raines A, Raymond GJ, Caughey B, Chesebro B. Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J Virol 2001; 75:10106-12.
15. Thackray AM, Klein MA, Aguzzi A, Budjoso R. Chronic subclinical prion disease induced by low-dose inoculum. J Virol 2002; 76:2510-7.

16. Thackray AM, Klein MA, Budjoso R. Subclinical prion disease induced by oral inoculation. J Virol 2003; 77:7991-8.
17. Collins SJ, Lewis V, Brazier MW, Hill AF, Lawson VA, Klug GM, Masters CL. Extended periods of asymptomatic prion disease after low dose inoculation: assessment of detection methods and implications for infection control. Neurobiol Dis 2005; 20:336-46.
18. Baier M, Norley S, Schultz J, Burwinkel M, Schwarz A, Riemer C. Prion diseases: infectious and lethal doses following oral challenge. J Gen Virol 2003; 84:1927-9.
19. Asante EA, Linehan JM, Gowland I, Joiner S, Fox K, Cooper S, et al. Dissociation of pathological and molecular phenotype of variant Creutzfeldt-Jakob disease in transgenic human prion protein 129 heterozygous mice. Proc Natl Acad Sci USA 2006; 103:10759-64.
20. Asante EA, Linehan JM, Desbruslais M, Joiner S, Gowland I, Wood AL, et al. BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. EMBO J 2002; 21:6358-66.
21. Castilla J, Saá P, Soto C. Detection of prions in blood. Nature Med 2005; 11:982-5.
22. Langeveld JPM, Jacobs JG, Erkens JHF, Bossers A, van Zijderveld FG, van Keulen LJM. Rapid and discriminatory diagnosis of scrapie and BSE in retropharyngeal lymph nodes of sheep. BMC Vet Res 2006; 2:19.
23. Soto C, Anderes L, Suardi S, Cardone F, Castilla J, Frossard M-J, et al. Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. FEBS Letts 2005; 579:638-42.
24. Foster JD, Parnham D, Chong A, Goldmann W, Hunter N. Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats. Vet Rec 2001; 148:165-71.
25. Goldmann W, Hunter N, Smith G, Foster J, Hope J. PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. J Gen Virol 1994; 75:989-95.
26. Diringer H, Roehmel J, Beekes M. Effect of repeated oral infection of hamsters with scrapie. J Gen Virol 1998; 79:609-12.
27. Gravenor MB, Stallard N, Curnow R, McLean AR. Repeated challenge with prion disease: the risk of infection and impact on incubation period. Proc Natl Acad Sci USA 2003; 100:10960-5.
28. Jacguemot C, Cuche C, Dormont D, Lazarini F. High incidence of scrapie induced by repeated injections of subinfectious prion doses. J Virol 2005; 79:8904-8.
29. Castilla J, Brun A, Segundo FD-S, Salguero FJ, Gutiérrez-Adán A, Pintado B, et al. Vertical transmission of bovine spongiform encephalopathy prions evaluated in a transgenic mouse model. J Virol 2005; 79:8665-8.
30. Wilesmith JW, Ryan JMB. Absence of BSE in the offspring of pedigree suckler cows affected by BSE in Great Britain. Vet Rec 1997; 141:250-1.